OPTIMAL COLLECTION OF BLOOD SAMPLES FOR THE MEASUREMENT OF TUMOR NECROSIS FACTOR α

Andrew R. Exley and Jonathan Cohen*

We have examined how delayed separation of plasma from cells affects the recovery of recombinant human tumor necrosis factor α (rhTNFα) from whole blood. Storage of heparinized whole blood samples at room temperature for 1 hr results in a significant (p = 0.036) fall in recovery of plasma TNFα from 788 ± 119 pg/mL to 472 ± 77 pg/mL, measured by specific enzyme-linked immunosorbent assay (ELISA). Storage of whole blood samples at 4°C for 1 hr reduces but does not prevent the fall in recovery of plasma TNFα: 725 ± 82 pg/mL at time 0, 472 ± 81 pg/mL after 1 hr, p = 0.038. Recovery of bioactive TNFα (cytotoxocity for L929 cells) after 1 hr at room temperature is also significantly reduced from 576 ± 139 pg/mL to 450 ± 154 pg/mL, p = 0.036. Studies with ^{125}I-rhTNFα confirmed the fall in plasma activity and revealed a rapid commensurate increase in ^{125}I-rhTNFα activity in the cell fractions. We recommend that clinical samples for the measurement of cytokines should be kept at 4°C and separated rapidly (within half an hour) before storing the plasma at −70°C.

Accurate, reliable measurement of tumor necrosis factor α (TNFα) in biological fluids is dependent both on the sensitivity and specificity of the assay and the recovery of TNFα from the initial sample. The stability of recombinant human TNFα (rhTNFα) in solution or in a lyophilized form has been well described,[1] but the optimal collection of blood samples has been little emphasized. Endotoxin contamination of commercially prepared tubes may stimulate the release of TNFα from heparinized blood resulting in false positive plasma samples with high levels of TNFα.[2] In contrast, false negative results may account for the failure to demonstrate TNFα in some patients with septicaemia[3] or cancer-associated cachexia.[4,5] We have examined the effects of delayed separation of plasma from cells on the recovery of rhTNFα from whole blood samples and the implications for measurement of TNFα in clinical samples.

RESULTS

Recovery of TNFα from spiked, heparinized whole blood samples decreases with time if separation of the plasma from the cells is delayed. When spiked, heparinized whole blood was kept at room temperature for 1 hr, mean plasma TNFα measured by specific enzyme-linked immunosorbent assay (ELISA), decreased from 788 ± 119 pg/mL at time zero, to 472 ± 77 pg/mL, p = 0.036 (Fig. 1).

A similar decline in plasma TNFα occurred when spiked, heparinized whole blood was kept at 4°C for 1 hr: mean plasma TNFα decreased from 725 ± 82 pg/mL at time zero to 473 ± 81 pg/mL, p = 0.036. However, when parallel spiked whole blood samples were held at room temperature or 4°C for 2 hr the mean fall in plasma TNFα was 452 ± 94 pg/mL (room temperature) compared with 313 ± 49 pg/mL (4°C), p = 0.036.

The decrease in plasma TNFα after delayed separation of plasma aliquots from whole blood was confirmed using the L929 assay (Fig. 2). When spiked, heparinized whole blood was kept at room temperature for 1 hr, plasma TNFα decreased from 576 ± 139 pg/mL at time zero to 450 ± 154 pg/mL, p = 0.036.

The plasma TNFα samples stored at −70°C and analyzed at 1, 4, and 16 weeks by the TNFα ELISA remained stable, varying by less than 10% with mean values of 1,228 ± 91 pg/mL, 613 ± 41 pg/mL, 262 ± 21 pg/mL, and 123 ± 12 pg/mL.

In the whole blood samples spiked with ^{125}I-rhTNFα, delayed plasma separation resulted in a rapid decline in the plasma radioactivity, in a fashion similar

Infectious Diseases Unit, Departments of Bacteriology and Medicine, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK.
*To whom correspondence should be addressed.

1043-4666/90/0205-0003$5.00/0

KEY WORDS: TNFα/Plasma/ELISA/Bioassay

CYTOKINE, Vol. 2, No. 5 (September 1990: 353-356)

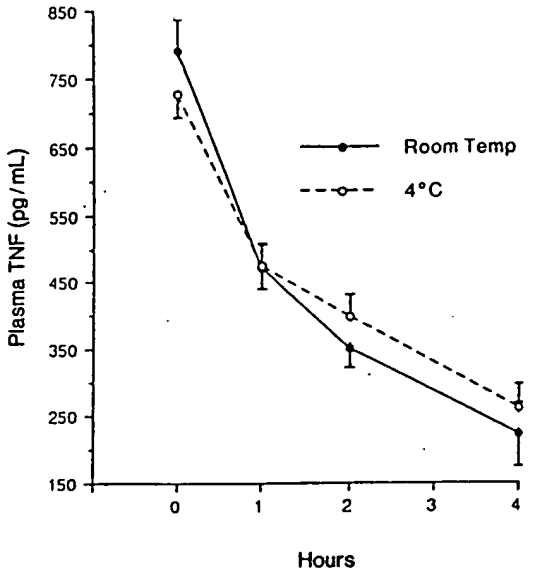

Figure 1. Change in plasma TNFα after delayed separation from whole blood: TNFα ELISA.

The figure shows the decline in plasma immunoreactive TNFα when whole blood samples are kept at room temperature or 4°C prior to plasma separation (mean and standard error of the mean, n = 6).

to that in the experiments using unlabeled TNFα. The fall in plasma ^{125}I-rhTNFα activity was confirmed by the TNFα ELISA, which showed a parallel decrease in immunoreactive ^{125}I-rhTNFα. Mirroring this decline in plasma ^{125}I-rhTNFα activity there was a rapid increase in ^{125}I-rhTNFα radioactivity in the cell fractions, (Fig. 3).

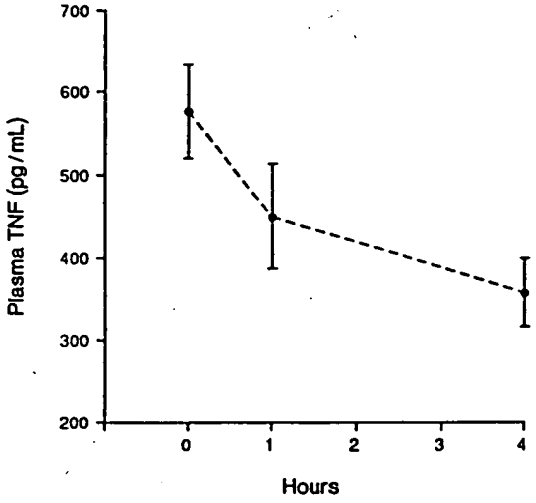

Figure 2. Change in plasma TNFα after delayed separation from whole blood: L929 assay.

The figure shows the decline in plasma TNFα bioactivity (as measured by the L929 cytotoxicity assay) when whole blood samples are kept at room temperature prior to plasma separation (mean and standard error of the mean, n = 6).

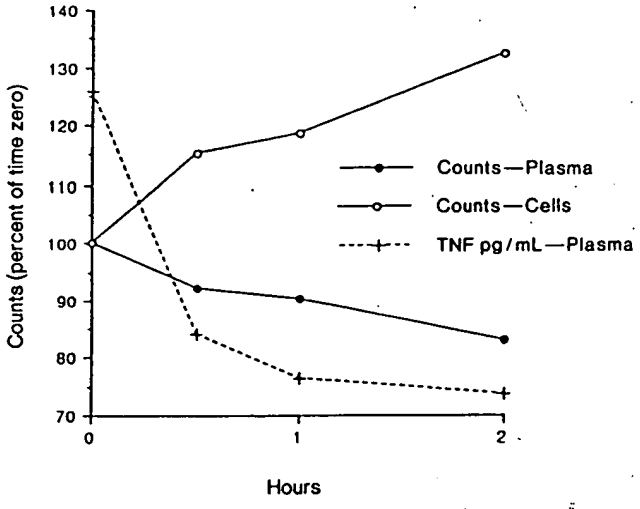

Figure 3. Change in plasma ^{125}I-rhTNFα activity with delayed separation from whole blood.

The figure shows the change in ^{125}I-rhTNFα radioactivity in the plasma and cell fractions relative to time zero and the fall in immunoreactive plasma ^{125}I-TNFα in pg/mL as determined by TNFα ELISA; the data shown are from a single representative experiment.

DISCUSSION

We have shown that recovery of rhTNFα from spiked, heparinized whole blood samples falls significantly if separation of plasma from cells is delayed. We have reported previously[6] that the loss of immunoreactive plasma TNF occurs at a range of concentrations similar to those reported in patients with severe meningococcal sepsis or falciparum malaria[3,7] and have shown here, using the L929 bioassay, that TNFα bioactivity is also lost. Storage of whole blood samples at 4°C prior to separation decreases but does not prevent this loss of measureable plasma TNFα.

The loss of TNFα during the recovery of plasma TNFα from the initial whole blood samples is far greater than any subsequent loss of activity during storage at −70°C. The plasma TNFα concentration determined by TNF ELISA varied by less than 10% over 4 months for aliquots stored at −70°C, and there is no significant loss of TNFα bioactivity (L929 cytotoxicity assay) over 9 months when rhTNFα in solution is stored at −70°C.[1]

To determine whether this loss of TNFα activity is due to biodegradation, biochemical modification, or binding to plasma proteins or cellular receptors, we carried out a number of studies with ^{125}I-rhTNFα. We have shown that the disappearance of ^{125}I-rhTNFα activity from the plasma fraction is temporally associated with a shift of radioactivity to the cellular fraction. High-affinity TNFα receptors are well described on peripheral blood leucocytes[8,9] but are not present on erythrocytes. It is likely, therefore, that our findings can be explained by the binding of TNFα to specific recep-

tors on leukocytes. These data suggest that this effect could potentially be an important cause of false negatives in blood samples analyzed for TNFα by immunoassays or bioassays. Furthermore, poor recovery of TNFα from the initial blood sample is a phenomenon that could be common to many of the other cytokines. In conclusion, we recommend that clinical samples for the measurement of cytokines should be stored at 4°C and separated rapidly (within half an hour) before storing the plasma at −70°C.

MATERIALS AND METHODS

Blood Sampling

Clinical grade sodium heparin (containing <50 pg/mL endotoxin by the *Limulus* amoebecyte lysate microassay[10]) was added to pyrogen-free plastic tubes (Sterilin, Feltham England) to give a final concentration of 10 IU heparin/mL blood. Fresh whole blood from healthy volunteers was collected into the tubes, spiked with rhTNFα (BASF, Ludwigshaven, West Germany) at a nominal concentration of 500 pg/mL whole blood, and mixed by repeated inversion. Paired blood samples were kept at room temperature and 4°C respectively and plasma aliquots withdrawn at time 0, 1, 2, and 4 hr, after centrifugation at 500*g* for 10 min. Plasma aliquots were frozen and stored at −70°C prior to assay by TNFα ELISA and L929 bioassay.

In other experiments samples were spiked with rhTNFα at concentrations of 1,000, 500, 250, and 125 pg/mL whole blood, mixed by inversion, then centrifuged promptly at 500*g* for 10 min. The plasma was withdrawn, aliquoted, and then analyzed by TNFα ELISA after storage of aliquots at −70°C for 1, 4, or 16 weeks. The mean and standard deviation for each TNFα concentration were calculated for all values.

TNFα ELISA

Plasma TNFα was measured by use of a modified TNFα-specific ELISA.[11] Briefly, 96-well plates (Nunc Immunotype 1, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0.25 μg/mL of the neutralizing murine IgG monoclonal antibody to rhTNFα, CB0006 (formerly 61E71, Celltech, Slough) in 0.05 M carbonate buffer and blocked by incubation at room temperature with 1% bovine serum albumin in phosphate-buffered saline. Test samples were added in triplicate and the standard titration curve obtained by serial doubling dilutions of rhTNFα in heat-treated normal human plasma. Bound TNFα was measured by sequential incubation with polyclonal rabbit anti-rhTNFα antibody (gift from W. Buurman, University of Limburg, Maastricht) and a goat anti-rabbit horseradish peroxidase conjugate antibody (Jackson, West Grove, PA, USA) followed by substrate (orthophenylenediamine, Sigma, St Louis, MO, USA). The color reaction was terminated with 1.0 M sulfuric acid and extinction measured at 492 nm with an automated Micro ELISA reader (Titertek Multiscan Plus MkII, Flow, Irvine). Values were derived from a standard curve of rhTNFα diluted in pooled, heat-treated plasma.

L929 Bioassay

A modified 3-day L929 assay was used[12] with confluent L929 murine fibrosarcoma cells (gift from F. Balkwill, ICRF, London) prepared in RPMI 1640 with 10% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin/Fungilin. Cell suspension, 100 μl/well at 3×10^5/mL, was added to 96-well microtiter plates (Falcon 3072 Microtiter III, Becton Dickinson, Lincoln Park, NJ, USA) and incubated at 37°C with 6% CO_2 for 20 hr. Twenty-five microliters of actinomycin D was added to each well to a final concentration of 1 μg/mL. Test plasma samples were heat treated at 56°C for 30 min and then centrifuged at 13,000 rpm to sediment platelets. Samples and serial dilutions of standard rhTNFα were added in quadruplicate to the L929 cells and placed in a humid incubator at 37°C with 6% CO_2 for 24 hr. The medium was discarded and the cells fixed in 5% formyl saline for 10 min and stained with fresh, filtered 0.5% crystal violet for 5 min. The plates were rinsed thoroughly in tap water and blotted dry before reading at 580 nm using an automated plate reader (Titertek Multiscan). Plasma TNFα values were derived by regression from standard curves of the change in mean optical density (mean optical density of plasma controls − mean optical density of TNFα standards) plotted against $\log_{10}$ standard rhTNFα concentrations. The L929 TNFα cytotoxicity assay applied to plasma aliquots from spiked whole blood produces TNFα levels lower than those produced by the TNFα ELISA, mean value 82% (95% confidence interval, 52 to 122%).[13]

Studies with Iodinated TNFα

^{125}I-rhTNFα, prepared using the Iodogen method,[14] was purified on a Sephadex G25M column (Pharmacia, Uppsala, Sweden) such that 95% of the radioactivity was precipitable by 15% trichloroacetic acid. In the TNFα ELISA, doubling dilutions of the ^{125}I-rhTNFα gave a titration curve parallel to that for unlabeled rhTNFα with a specific activity of 12×10^7 cpm/μg TNFα. Fresh heparinized blood samples were spiked with 300 pg rhTNFα (BASF) per mL of blood or ^{125}I-rhTNFα at 2,500 cpm/mL, mixed thoroughly by inversion, and aliquoted at time zero. Samples were stored at room temperature or 4°C for intervals before centrifugation and separation of the plasma. ^{125}I-rhTNFα activity in plasma and cell fractions was counted for 60 sec in a γ counter and the amount of ^{125}I-rhTNFα in each fraction was expressed as a percentage of activity at time zero, corrected for the total activity in each aliquot. The ^{125}I-rhTNFα levels in the plasma fractions were also determined by the TNFα ELISA.

Statistical Methods

The Wilcoxon signed-rank test was used to compare paired plasma TNFα samples. All tests were two-sided.

Acknowledgements

This work was supported in part by a grant from Celltech Limited, Slough, England. J.C. is a Wellcome Senior Lecturer in Infectious Diseases.

REFERENCES

1. Geigert J, Panschar BM, Taforo C, Paolo J, Fong S, Huston HN, Wong DE, Wong DY (1988) Parameters for the evaluation of long-term stability of tumour necrosis factor preparations. Dev Biol Stand 69:129-138.

2. Leroux-Rouls G, Offner F, Phillipe J, Vermeulen A (1988) Influence of blood-collecting systems on concentrations of tumour necrosis factor in serum and plasma. Clin Chem 34:2373-4.

3. Waage A, Halstensen, Espevik T (1987) Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. Lancet i:355-7.

4. Balkwill F, Burke F, Talbot D, Tavernier J, Osborne R, Naylor S, Durbin H, Fiers W (1987) Evidence for tumour necrosis factor/cachectin production in cancer. Lancet ii:1229-1232.

5. Socher SH, Martinez D, Craig JB, Kuhn JG, Oliff A (1988) Tumor necrosis factor not detectable in patients with clinical cancer cachexia. J Natl Cancer Inst 80:59-62.

6. Exley AR, Cohen J (1989) Recovery of recombinant human tumour necrosis factor from whole blood—implications for measurement in clinical samples [Abstract]. Cytokine 1:137

7. Grau GE, Taylor TE, Molyneux ME, Wirima JJ, Vassali P, Hommel M, Lambert PH (1989) Tumor necrosis factor and disease severity in children with falciparum malaria. N Engl J Med 320:1586-1591.

8. Shalaby MR, Paladino MA, Hirabayashi SE, Eessalu TE, Lewis GD, Shepard HM, Aggarwal BB (1987) Receptor binding and activation of polymorphonuclear neutrophils by tumour necrosis factor-alpha. J Leukocyte Biol 41:196-204.

9. Hohmann HP, Remy R, Brockhaus M, van Loon, APGM (1989) Two different cell types have different major receptors for human tumor necrosis factor (TNFα). J Biol Chem 264:14927-14934.

10. Cohen J, McConnell J (1984) Observations on the measurement and evaluation of endotoxemia by a quantitative Limulus lysate microassay. J Infect Dis 150:916-924.

11. Debets JMH, van der Linden CJ, Spronken IEM and Buurman W (1988) T cell mediated production of tumour necrosis factor-a by monocytes. Scand J Immunol 27:601-608.

12. Fomsgaard A, Worsaae H, Bendtzen K (1988) Detection of tumour necrosis factor from lipopolysaccharide-stimulated human mononuclear cells by enzyme-linked immunosorbent assay and cytotoxicity bioassay. Scand J Immunol 27:143-147.

13. Bland JM, Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. Lancet i:307-310.

14. Fraker PJ, Speck JC (1978) Protein and cell membrane iodinations with sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenyl-coluril. Biochem Biophys Res Commun 80:849-857.